

1 Interaction of coffee compounds with serum 2 albumins. Part II: diterpenes

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7 ABSTRACT

8 Cafestol and 16-*O*-Methylcafestol are diterpenes present in coffee, but while cafestol is found in
9 both *Coffea canephora* and *Coffea arabica*, 16-*O*-methylcafestol (16-OMC) was reported to be
10 specific of the only *C. canephora*. The interactions of such compounds with serum albumins have
11 been studied. Three albumins have been considered, namely human serum albumin (HSA), fatty
12 acid free HSA (ffHSA) and bovine serum albumin (BSA). The proteins interact with the diterpenes
13 at the interface between Sudlow site I and the fatty acid binding site 6 in a very peculiar way,
14 leading to a significant change in the secondary structure. The diterpenes do not displace reference
15 binding drugs of site 2, but rather they enhance the affinity of the site for the drugs. They, therefore,
16 may alter the pharmacokinetic profile of albumin – bound drugs.

17 KEYWORDS

18 Coffee; Human serum albumin; Bovine serum albumin; Fluorescence spectroscopy; diterpenes

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ABBREVIATIONS

HSA, human serum albumin; ffHSA, fatty acid – free HSA, BSA, bovine serum albumin; PBS, phosphate buffer solution; 16OMC, 16-*O*-methylcafestol; DMSO, dimethyl sulfoxide; Trp, tryptophan

1. INTRODUCTION

Coffee is undoubtedly one of the most consumed and appreciated beverage in the world. The two commercially exploited species of coffee, *Coffea arabica* (arabica) and *Coffea canephora* (var. Robusta), have been extensively studied so far as chemical composition is concerned particularly for sensory, traceability and authenticity purposes. However there is still a great attention in understanding the chemical properties of coffee major constituents and their biological effects as witnessed by the body of articles appearing in the literature every year. The genome code of *Coffea canephora* has just been sequenced giving origin to new frontiers in the comprehension of the biosynthesis of coffee secondary metabolites (Denoued et al., 2014). The chemical composition of coffee beans depends on both on the coffee species (*arabica* or *robusta*) and on the geographical region of the cultivars (Kitzberger et al., 2013), on the roasting process (Eloy Dias, Ferreira, Zerlotti Mercadante, Bragagnolo & de Toledo Benassi, 2014) and on the method used to prepare the coffee beverage. All these variables influence the perceived sensory properties of coffee brands and for this reason industry pays great attention to monitor all these factors.

Among the vast array of compounds present in coffee brew, the biological active classes are usually considered to be the phenolic compounds (chlorogenic acids), the alkaloids caffeine and trigonelline, the diterpenes cafestol and kahweol and melanoidins. These compounds have been shown, at least in vitro, to possess various properties including antioxidant, chemopreventive,

antihypertensive and hypoglycemic activity. A recent review by Croizer et al. considered the overall literature regarding the potential impact on health of the phytochemicals present both in green and roasted coffee beans (Ludwig, Clifford, Lean, Ashihara & Croizer A. 2014). The diterpene alcohols of the kaurene family, cafestol, kahweol and 16-*O*-methylcafestol (fig. 1) are produced only by plants of the *Coffea* genus, but while cafestol was found in both *C. arabica* and *C. canephora*, kahweol is present in *C. arabica* in large amounts, while only traces are found in *C. canephora*. On the contrary, 16-*O*-methylcafestol was found to be specific to *C. canephora*. The total diterpene content ranges from 1.3% to 1.9% (w/w) in green coffee beans of *Coffea arabica* and from 0.2% to 1.5% in beans of *Coffea canephora*. In particular, diterpenes have been extensively studied and show beneficial effects to human health as anti-inflammatory properties, a prevention on DNA damage from oxidative stress, although a hypercholesterolemic effect attributed to cafestol was also observed (Bonita, Mandarano, Shuta, & Vinson, 2007).

To better understand the biological impact on human health of these compounds, a study of their interaction with human serum albumin (HSA) is recommended since albumin is the most abundant protein in human plasma. It is a monomeric 585-residue protein containing three homologous helical domains (I-III), each divided into two subdomains (A and B) (He & Carter, 1992). Two main binding sites for small organic molecules are found, one located in subdomain IIA and one in IIIA, that are known as Sudlow I and Sudlow II sites, respectively (Sudlow, Birkett & Wade, 1975a).

Bovine serum albumin (BSA) has been extensively studied in kinetic and affinity drug tests as a replacement for human serum albumins (HSA) because of its easy accessibility, high stability, ability to bind various ligands and structural similarity to HSA (Shinga Roy, Tripathy Chatterjee

& Dasgupta, 2010; Zhang et al., 2013). The structure of BSA is homologous to HSA and consists of three linearly arranged domains (I-III) that are composed of two subdomains (A and B).

In our previous study we have determined by fluorescence spectroscopy the dissociation constants for the complexes of chlorogenic acids and quinides with HSA, which were in the micromolar range (Sinisi et al., 2015).

In the present work we have considered the diterpenes alcohols cafestol and 16-*O*-methylcafestol, isolated from commercial *Coffea canephora* blends and we have studied their interactions with albumins by fluorescence and circular dichroism spectroscopies.

2. MATERIALS AND METHODS

2.1 Materials

HSA (A1653, 96-99%), HSA essentially fatty acid free (A3782, 99%), BSA (A3912, $\geq 96\%$) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) and used without further purification. Their molecular weight were assumed to be 66.478 Da, 66.478 Da and 66.463 Da respectively. Stock solutions of albumins were prepared by dissolving it in PBS (pH 7.4). All stock solutions were kept at 4 °C and then diluted to the required experimental sample concentrations (1.0×10^{-6} M). Cafestol and 16OMC were provided by Illycaffè S.p.A. (AromaLab, TS, Italy). Cafestol and 16OMC stock solutions (1.25 mM, 2.5 mM e 5 mM) were prepared in DMSO.

2.2 Fluorescence Spectroscopy

All steady-state fluorescence spectra were recorded at 25 °C on a *CARY Eclipse* (Varian) spectrofluorimeter equipped with a 0.5 cm path length quartz cuvette. An excitation wavelength

of 280 nm (λ_{exc}) was used in all cases for selective excitation of the Trp residues of albumins, and emission spectra were recorded from 300 to 400 nm. For synchronous fluorescence spectra (SFS), $\Delta\lambda$ (the constant wavelength interval between the emission and the excitation wavelength) was set at 60 nm, and the SFS were recorded from 240 to 320 nm. The slit width on the excitation was set to 10 nm, on the emission to 10 nm. Quenching experiments were performed by keeping the concentration of albumins fixed at 1 μM in 350 μL of solvent (135 μL of phosphate buffer 10 mM in Na_2HPO_4 and 2 mM in KH_2PO_4 diluted in 215 μL of mQ water, pH 7.4) for all the measurements; diterpenes concentrations varied from 0 to 500 μM by adding aliquots of their stock solutions. The final amount of DMSO was always 10%, and it has been verified that such amounts of solvent do not affect the fluorescence of albumins. After each addition of the ligand, the emission spectra, the fluorescence intensity, and the SFS were recorded. All the analyses were replicated three times.

2.3 Warfarin displacement studies

The displacement of warfarin was studied with the same spectrofluorimeter and cell, in the same buffer described above for the binding study. Warfarin was added to the buffer at a 10 μM final concentration from a 1 mM reference solution in DMSO. HSA was then added at a 1 μM final concentration and the emission spectrum was recorded upon excitation of bound warfarin at 320 nm. The emission maximum was observed at 380 nm. Cafestol and 16OMC were then added at increasing concentrations by adding aliquots of its stock solution in the 5-500 μM range, and the emission spectrum was recorded again at each addition.

2.4 Circular dichroism

All titrations were performed at room temperature on a *Jasco J-715 Spectropolarimeter* equipped with a 0.1 cm path length quartz cuvette. A wavelength range of 190-380 nm was selected and a scan speed of 50 nm/min was chosen. Cafestol and 16OMC were dissolved in 1 mL of methanol to give a 1.5 mM solution. Titrations were performed by keeping the concentration of albumins (HSA and ff-HSA) fixed at 5 μ M in 500 μ L of solvent (135 μ L of phosphate buffer 10 mM in Na_2HPO_4 and 2 mM in KH_2PO_4 diluted in 215 μ L of mQ water, pH 7.4) for all the measurements; diterpenes concentrations varied from 0 to 100 μ M by adding aliquots of their stock solutions (0, 1, 5, 10, 20, 40, 60, 80, 100 μ M). After each addition of the ligands, a CD spectrum was recorded.

3. RESULTS AND DISCUSSION

As outlined in the introduction, both HSA and BSA have two main binding sites, the Sudlow site I in subdomain IIA and the Sudlow site II in subdomain IIIA, which differ in shape, size and polarity, and therefore in their binding specificity (Ghuman et al., 2005). A major characteristic of Sudlow site I is the presence of a tryptophan residue (Trp214 in HSA, 213 in BSA) within it. BSA has also another tryptophan at position 134. This second residue is buried inside a small hydrophobic pocket near the surface of the protein, in the second helix of the first domain, far from the main binding sites of the protein for small drugs and fatty acids. Tryptophan is fluorescent and if it is excited at around 280 nm, an emission maximum close to 340 nm is often observed; this maximum may vary from 310 nm to 350 nm, depending on the electronic environment of the indole system (Adams et al., 2002). A molecule able to bind inside the Sudlow site I of albumins causes often a change in the emission of Trp214 resulting in either an enhancement or in a quenching phenomenon depending on the way the environment surrounding the residue is altered upon binding. Fluorescence titrations were performed to study the interactions of cafestol and 16-

132 OMC with BSA, HSA and ffHSA. Commercial source albumins are in fact fatty-acid bound and
133 almost all the fatty acid binding sites are occupied. ffHSA is used as a reference as it is known that
134 the occupancy of the fatty acids binding sites may change the affinity of the protein for the drug
135 binding sites, mostly for the Sudlow site I which is contiguous to the myristic acid site FA6: in this
136 case tyrosine 210 is turned towards the fatty acid carboxylic head when the FA6 site is occupied,
137 to establish a hydrogen bond with the carboxylate, while is turned towards the drug site in the
138 absence of fatty acids (Figure 2A). In all the measurements, the concentration of protein was 1
139 μM in 350 μL of solvent, obtained by diluting 135 μL of 10 mM Na_2HPO_4 and 2 mM KH_2PO_4
140 phosphate buffer with 215 μL of mQ water; the pH was 7.4. The ligand concentration was
141 gradually increased during the titration from 5 μM to 500 μM using ligand standard solutions in
142 DMSO. The emission (λ_{exc} 280 nm, λ_{em} range 300-400 nm) spectra of the protein alone were
143 recorded at the beginning of any experiments. After each addition of the ligand, the emission
144 spectra were monitored. Two examples of the resulting spectra are reported in **fig. 3**, while the
145 others are reported in the supplementary data. The emission spectra of the three proteins undergo
146 major changes upon addition of the two diterpenes, and the general trend is very unusual. With 16-
147 OMC, fluorescence quenching is observed in all the experiments at low concentrations of the
148 added ligand, and a very large blue shift also occurs at the beginning, where the maximum emission
149 wavelength is shifted by 15 – 20 nm and over (see the inserts in fig.3). With 16-OMC the shift
150 occurs up to 40 μM final concentration, and after this point the maximum wavelength remains
151 almost constant. The addition of cafestol leads also to a similar shift, but with human albumins an
152 increase of emission rather than a quenching occurs (**fig. 3B**), while with BSA a large quenching
153 is again observed.

We have evaluated the effect of the ligands on the protein emission by plotting the normalized emission spectrum area versus the concentration of added diterpenes (Fig. 4). The emission of the proteins undergoes to a rapid change (either quenching or increase) at the beginning, and this phenomenon ends at the same concentrations of diterpenes at which the maximum emission wavelength reaches its lower plateau. After this point the emission undergoes a further, slight and irregular quenching with 16OMC in all the proteins, and BSA is the most sensitive while HSA and ffHSA give almost superimposable results. The quenching induced by cafestol on BSA is of the same extent of that obtained with 16OMC, while the emission of HSA is enhanced more than that of ffHSA, and in a wider range of ligand concentration. In order to explain the observed behaviour we have considered first the low concentration region of the plot in fig. 4. We have run first synchronous spectra at $\Delta = 60$ nm at the low diterpene concentrations (see supplementary data), and we have verified that the whole of the observed quenching / enhancement in the emission spectra is observed also in the corresponding synchronous ones. As the synchronous spectra allow to selectively record tryptophan emissions and avoid to collect also emissions from tyrosine, this experiment confirms that the change in emission is mostly due to tryptophan quenching. We have then analyzed the low concentration using the Stern-Volmer equation (**equation 1**) that describes the quenching process:

$$\frac{F_0}{F} = 1 + K_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad \text{Eq. 1}$$

The variables F_0 and F are the emission intensities before and after the addition of the quencher, respectively, K_q is the bimolecular quenching kinetic constant, i.e. a collisional frequency between freely diffusing molecules, τ_0 is the lifetime of the fluorophore - for the tryptophan fluorescence decay τ_0 is about 10^{-8} s (Valensin, Kushnir & Navon, 1982; Krag-Hansen, 1990)- K_{SV} is the Stern-Volmer quenching constant and $[Q]$ is the quencher concentration in mol/L; the protein

concentration was fixed to 1 μ M. The K_{SV} for the two ligands were determined by linear regression of a plot of F_0/F against $[Q]$ (see supplementary data) in the low ligand concentration range, where all the plots were linear. K_{SV} and K_q (calculated using the equivalence $K_q = K_{SV}/\tau_0$) are reported in **table 1**.

The bimolecular quenching kinetic constants (K_q) are 1-2 orders of magnitude higher than the maximum value for diffusion-limited collisional quenching ($2.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$) (Eftink, 1991; Ware, 1962), thus the static quenching originating from the association of the fluorophore and quenchers in a bimolecular complex is the main contribution to the fluorescence quenching mechanism in the low ligand concentration range. K_{SV} can be thus regarded as the association constant for the formation of the albumin – diterpene complexes. As to the effect of cafestol on the emission of human albumins, assuming that the enhancement of emission is linearly related to the fraction of occupied binding sites, we obtain apparent binding constants similar in value to those obtained with 16-OMC using the Stern – Volmer analysis. The order of magnitude of such constants, in the $10^3 - 10^4 \text{ L mol}^{-1}$ range, places our diterpenes among many other small molecules that are bound by albumins with similar affinities. However, they are far to being the best binders, and previous work carried out in our research group have shown that other coffee compounds as the phenolic family of chlorogenic acids and their quinide lactones are capable to bind to albumins with association constants in the order of $10^7 - 10^8 \text{ L mol}^{-1}$ (Sinisi et al., 2015). 16-OMC is bound by human albumins in a more favorable way than cafestol, and BSA appears to offer the best interaction way to both the diterpenes.

An interaction at the Sudlow site I is suggested by the observed effect on tryptophan fluorescence, as it is known that in human albumin similar effects are not observed with site II ligands, due to

the distance from the fluorophore. As to bovine albumin, the very similar amount of quenching suggests that the interaction occurs at the same site and involves the same tryptophan residue. It is known that the emission of the second tryptophan in BSA is low and not affected by ligand binding. In order to gain further information on the binding site area, we have setup a competition experiment with a reference ligand of Sudlow site I. We have chosen warfarin as this drug is the reference ligand of Sudlow site I; moreover, the intrinsic fluorescence of warfarin, which occurs at 320 nm excitation and 380 nm emission, is strongly enhanced by the interactions with albumin, and decreases upon competition with other drugs for the protein. This phenomenon has been exploited to set up a well-established method to study drug association to HSA (Sudlow, Birkett, & Wade, 1975a). The experiment was carried on a 10 μ M solution of warfarin in phosphate buffer, containing 1 μ M HSA. In the initial solution, the warfarin – albumin complex is formed, and this is confirmed by the fact that after the addition of albumin, the warfarin emission is enhanced by about 70%. To our surprise, the fluorescence emission of warfarin is further increased upon the addition of the diterpenes in the low concentration range (see supplementary material). The dissociation constant for the warfarin – albumin complex is reported to be 4 μ M, (Sudlow, Birkett & Wade, 1975b) thus under our initial conditions (albumin 1 μ M, warfarin 10 μ M) the fraction of albumin occupied binding sites is 70%. Due to the fact that there is still free albumin available in the system, we can explain the further enhancement in emission with an increase in the affinity of the protein for warfarin upon binding of the coffee diterpenes. This happens with both 16-OMC and cafestol, despite the fact that their different effect on albumin tryptophan emission (fig. 4) suggests a different mode of binding.

This behaviour is very unusual, as the Sudlow site I ligands displace warfarin by competition in the binding site, rather than enhancing the affinity. To our knowledge, an increased affinity is only

222 observed for warfarin in the presence of certain fatty acids.(Vorum & Honoré, 1996; Ni, Zhang
223 & Kokot, 2009) We therefore conclude that the binding site for our diterpenes is not exactly
224 corresponding to the central cavity of site I, but is rather involving also the fatty acid binding site.
225 Moreover we have to assume that in the terpene-warfarin-albumin ternary complex, tyrosine 150
226 is turned away from the site I core as it happens with fatty acids, and site I shifts to a more
227 favourable shape to interact with warfarin. An interaction occurring mostly at the very
228 hydrophobic fatty acid site 6 is on the other side in agreement with the lipid character of our
229 molecules, and with the more favourable interaction of 16-OMC in comparison with the more
230 polar headed cafestol. A preliminary model was built by docking 16-OMC cafestol inside binding
231 site 6 and the minor hydrophobic pocket of site I in the presence of warfarin. Two favourable
232 docking poses were found by AutoDock Vina (Trott & Olson, 2010) and are reported in fig. 2B.
233 In both the poses the “polar head” of the terpene is interacting with the polar aminoacids at the
234 border between site 6 and site I.

235 The shift in the maximum emission wavelength reach at the end of the low ligand concentration
236 range, and the subsequent lack of a further quenching / enhancement upon further additions of the
237 diterpenes is almost unprecedented in the literature as to the binding of small molecules to
238 albumins, while the only comparable result has been obtained, to our knowledge, with ionic
239 surfactants (Gelamo & Tabak, 2000). The large blue shift has been explained with a major
240 conformational change in the protein, involving also a change in the solvent exposition of the
241 fluorophore. In this hypothesis the addition of coffee diterpenes would lead to a change in the
242 secondary structure of the proteins. To study the potential structural change, we have recorded the
243 CD spectra of HSA and fhHSA in the presence of increasing amounts of diterpenes (an example is
244 reported in **fig. 5**). Although the spectra show an overall conservation of the secondary structure,

a significant decrease of the α -helix content can be observed by the increase of molar ellipticity at 222 nm (see supplementary data). The average decrease of α -helix content upon addition of diterpenes up to 100 μ M can be estimated at 10%. This decrease has to be compared with α -helix content of native serum albumins in solution, which is 57%. A similar result was observed by Gelamo and Tabak with surfactants as sodium dodecyl sulphate at concentrations exceeding 1 mM. The result seems to suggest that a partial change in the secondary structure of albumins occurs upon interaction with our diterpenes. It is known that in albumins the helical loops forming the domains, can associate to form a globular structure or separate reversibly, mostly by changing the environment pH, or the temperature. Changing the pH, five different conformational forms of HSA have been recognized: **F**, or Fast, at pH 4; **E**, or Expanded, below pH 3; **N**, or Normal, at neutral pH; **B**, or Basic, near pH 8 and **A**, or Aged, near pH 10. (Sugio et al., 1999) The Expanded form is the most elongated and disordered isomer; it is considered, in different works, as a reference of a completely unfolded albumin state, even if Muzammil et al. suggest that at pH 2.0, HSA resembles the molten globule state.(Muzammil, Kumar & Tayyab, 1999) More recently, a small angle X-Ray scattering study has allowed to clarify that the **E** form conserves a significant amount of domain folding, although its shape is expanded to a cigar-like one.(Leggio, Galantini & Pavel, 2008). The CD spectrum of the **E** form as recorded by Muzammil with ours shows that the amount of lost α -helical content in the **E** form is by far more than what occurs in our case, and we can possibly envisage our terpene-albumin complexes as an intermediate structure between form **N** and **E**, with the domains still folded but with a considerable exposition to the solvent of several of the inner aminoacids.

4. CONCLUSIONS

In summary, we have demonstrated that HSA and BSA are able to bind the coffee diterpenes in a very peculiar way, almost unprecedented in the recognition of small molecules by these proteins. The binding event is likely to occur at the interplay between the Sudlow drug site I and one of the fatty acid binding sites of the protein, which undergoes to a significant conformational change upon recognition of the diterpenes. This leads to a remarkable increase of the affinity of human albumin for a reference drug as warfarin, rather than to a competition for the drug. Dietary assumption of coffee diterpenes could therefore alter the pharmacokinetic profile of drugs binding to albumin.

5. ACKNOWLEDGMENTS

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6. SUPPLEMENTARY DATA

Emission spectra of ffHSA and BSA upon addition of 16-OMC and cafestol; synchronous spectra of HSA upon addition of 16-OMC, decrease of the emission spectra normalized integrals; Stern-Volmer plots for all the fluorescence experiments; increase of warfarin emission in the presence of HSA and of increasing concentrations of diterpenes.

7. FIGURE CAPTIONS

Fig. 1: structures of the ent-kaurene systems, of cafestol and 16-O-Methylcafestol.

Fig. 2A: outline of Sudlow site I and of fatty acid binding site 6 in human albumin. The reference ligand of site I, warfarin, is located inside the main hydrophobic pocket and shows also a phenyl

ring pointing towards the fluorescent side chain of tryptophan 214 in the front hydrophobic pocket. The three polar aminoacids Y150, R257 and H288 at the borderline between site I and site 6 are shown. Tyrosine 150 is turned towards the carboxylic head of a molecule of myristic acid. From pdb id 1H9Z. **2B**: AutoDock Vina calculated poses for 16-OMC in the binding areas of site I and 6, in the presence of warfarin. In the green solution the terpene is fully placed inside the fatty acid binding site, while in the red one the ligand is placed in the minor hydrophobic pocket of site I, but the hydroxyl group and its neighbours lie in site 6 close to the polar aminoacids.

Fig. 3A: emission spectra of 1 μ M HSA (1) upon addition of increasing amounts (2 – 20) of 16-OMC. The final concentrations of 16-OMC were 5, 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450 and 500 μ M in spectra 2 – 20 respectively. Insert: drift of the maximum emission wavelength upon increasing the concentration of 16-OMC. **3B**: emission spectra of 1 μ M HSA (1) upon addition of increasing amounts (2 – 14) of cafestol. The final concentrations of cafestol were 5, 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, and 200 μ M in spectra 2 – 18 respectively. Insert: drift of the maximum emission wavelength upon increasing the concentration of cafestol.

Fig. 4. normalized emission spectrum integrals (average of five repeated titrations) vs. the concentration of added terpene.

Fig. 5. Far UV circular dichroism spectra of ffHSA in the presence of increasing concentrations of 16-OMC at 0, 5, 10, 20, 40, 60, 80, 100 μ M in spectra 1-8 respectively.

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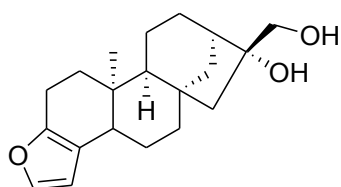
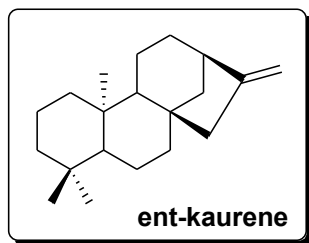
9. TABLES

Complex	$K_{SV} \pm SD \text{ (L mol}^{-1}\text{)}$	$K_q \text{ (L mol}^{-1} \text{ s}^{-1}\text{)}$
16-OMC - HSA	8100 ± 250	8.1×10^{11}
16-OMC - ffHSA	10200 ± 320	1.02×10^{12}

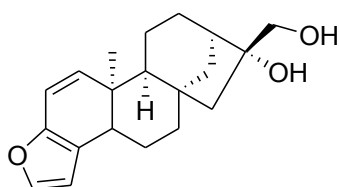
16-OMC - BSA	14300 ± 400	1.43×10^{12}
<i>Cafestol - HSA</i>	<i>5000 ± 110</i>	
<i>Cafestol - ffHSA</i>	<i>2460 ± 90</i>	
Cafestol - BSA	16000 ± 430	1.60×10^{12}

Table 1 Quenching constants according to Stern-Volmer analysis: Stern-Volmer quenching constant (K_{SV}) and bimolecular quenching kinetic constant (K_q). The binding constant for cafestol and human albumins reported in italics have been calculated in a similar way and they are the opposite of the slopes obtained in the Stern-Volmer analysis, assuming that the emission enhancement is linearly dependent from the cafestol concentration in this range. They should be regarded as an indication of a binding event rather than a Stern – Volmer constant.

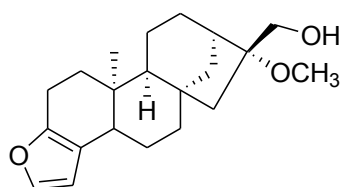
FIGURES



cafestol

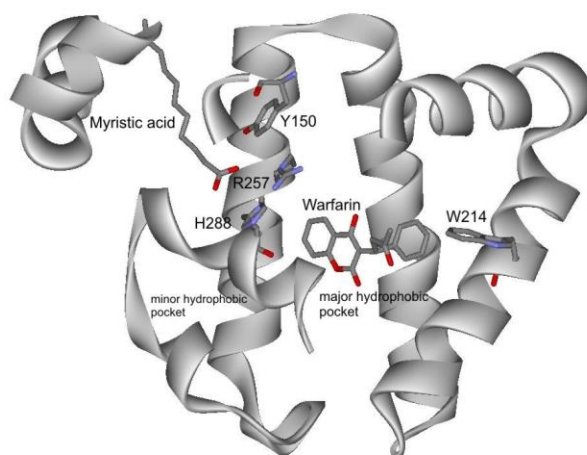


kahweol

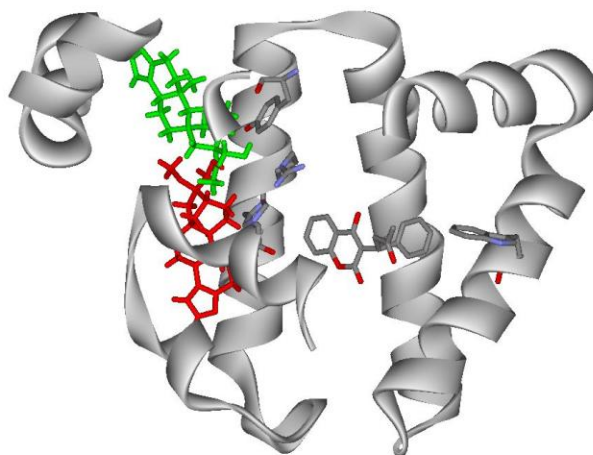


16-O-methylcafestol

Fig. 1

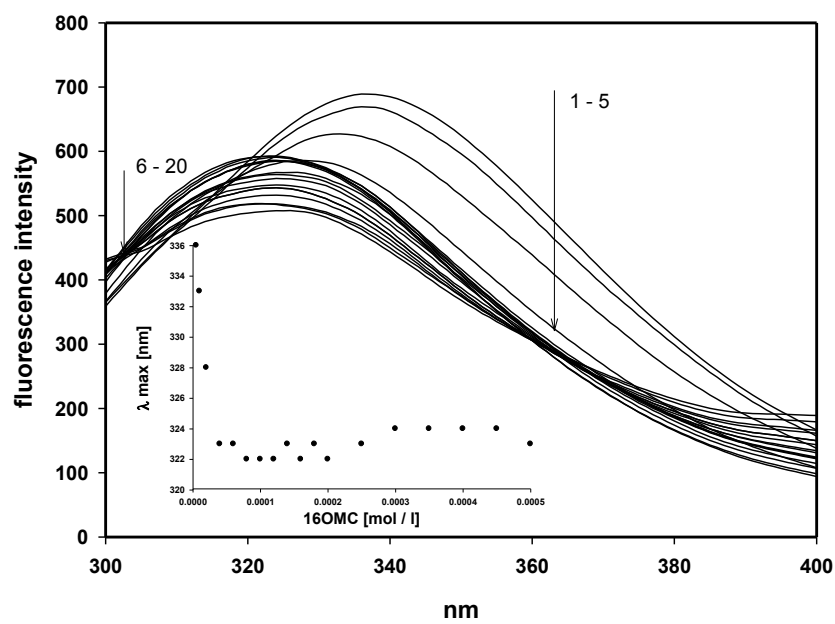


A

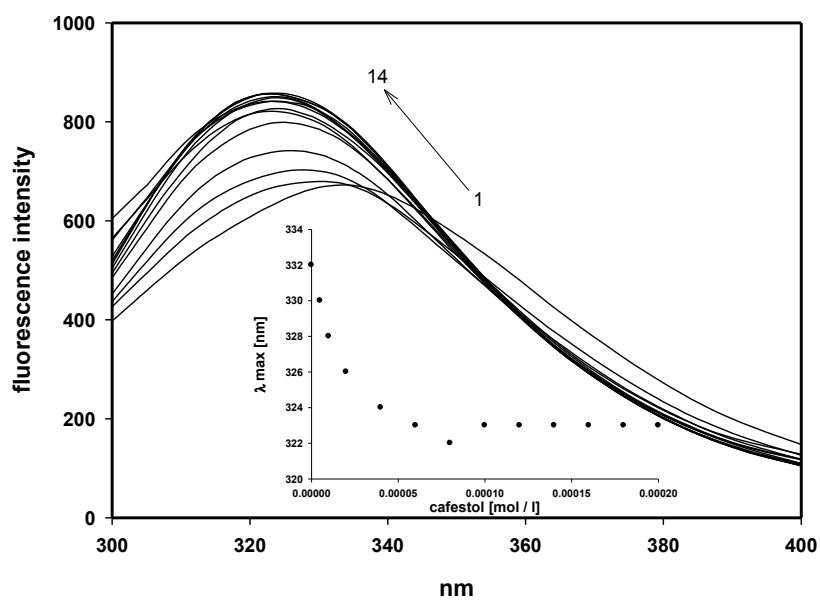


B

Fig. 2



A



B

Fig. 3

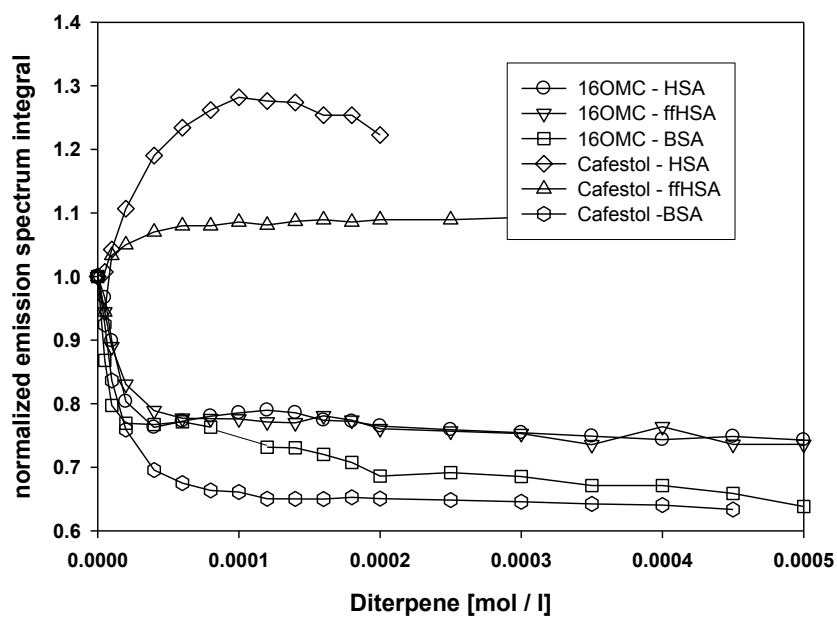
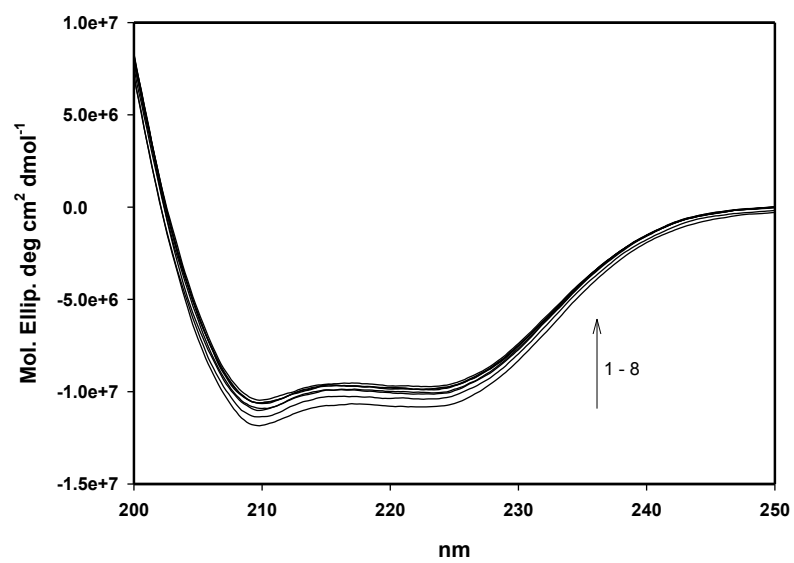


Fig. 4

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Fig. 5.

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